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Preactivation with IL-12, IL-15, and IL-18 Induces CD25 and a Functional High-Affinity IL-2 Receptor on Human Cytokine-Induced Memory-like Natural Killer Cells

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Natural killer (NK) cells are effector lymphocytes that are under clinical investigation for the adoptive immunotherapy of hematologic malignancies, especially acute myeloid leukemia. Recent work in mice has identified innate memory-like properties of NK cells. Human NK cells also exhibit memory-like properties, and cytokine-induced memory-like (CIML) NK cells are generated via brief preactivation with IL-12, IL-15, and IL-18, which later exhibit enhanced functionality upon restimulation. However, the optimal cytokine receptors and signals for maintenance of enhanced function and homeostasis after preactivation remain unclear. Here, we show that IL-12, IL-15, and IL-18 preactivation induces a rapid and prolonged expression of CD25, resulting in a functional high-affinity IL-2 receptor (IL-2Rαβγ) that confers responsiveness to picomolar concentrations of IL-2. The expression of CD25 correlated with STAT5 phosphorylation in response to picomolar concentrations of IL-2, indicating the presence of a signal-competent IL-2Rαβγ. Furthermore, picomolar concentrations of IL-2 acted synergistically with IL-12 to costimulate IFN-γ production by preactivated NK cells, an effect that was CD25 dependent. Picomolar concentrations of IL-2 also enhanced NK cell proliferation and cytotoxicity via the IL-2Rαβγ. Further, after adoptive transfer into immunodeficient NOD-SCID-$\gamma_c^{-/-}$ mice, human cytokine–preactivated NK cells expand preferentially in response to exogenous IL-2. Collectively, these data demonstrate that human CIML NK cells respond to IL-2 via IL-2Rαβγ with enhanced survival and functionality, and they provide additional rationale for immunotherapeutic strategies that include brief cytokine preactivation before adoptive NK cell transfer, followed by low-dose IL-2 therapy.

INTRODUCTION

Natural killer (NK) cells are a subset of innate lymphoid cells critical for host antiviral defense and mediate antitumor immunity [1-5]. NK cells are of clinical interest and are being explored as antitumor effectors in the allogeneic hematopoietic stem cell transplantation (HSCT) setting as well as an adoptive cellular therapy of hematologic disease [6-8]. Initial reports in the MHC-haploidentical transplantation setting indicated that NK cells may be harnessed for graft-versus-leukemia effects in the absence of graft-versus-host disease [9]. Subsequent studies have investigated the molecular basis of killer-cell immunoglobulin-like receptor genetics and their MHC class I ligands on NK cell functional responses and outcomes after allogeneic HSCT [10-12]. These studies highlight the importance of integrating new advances in basic NK cell biology, such as education and licensing, when applying NK cells as therapeutics in the HSCT or adoptive transfer setting.

NK cells are traditionally classified as innate immune lymphocytes because they do not rearrange germline DNA to form a dominant clonal activation receptor, distinct from T and B cells. However, this paradigm has recently been challenged by several groups identifying innate memory mediated by mouse NK cells [13] in the setting of hapten-based sensitization [14], viral (murine cytomegalovirus [MCMV]) infection [15], and after cytokine activation with IL-12, IL-15, and IL-18 [16]. Notably, NK cell memory that occurs after MCMV infection depends on proinflammatory cytokines [17], suggesting a common mechanistic link between virus- and cytokine-induced NK cell memory. Studies in humans have also shown that viral infection, in particular, human cytomegalovirus, results in imprinting on the NK cell compartment via altering the expression patterns of NKG2C and killer-cell immunoglobulin-like receptors that correlate with NK cell functional status. These studies include cytomegalovirus reactivation after solid organ transplantation and HSCT, which may correlate with murine virus-induced memory NK cells [18,19]. Human NK cell memory-like responses have been directly demonstrated in vitro after cytokine activation with IL-12, IL-15, and IL-18 [20]. A brief (16 hour) preactivation with IL-12, IL-15, and IL-18, followed by rest in vitro for 1 to 6 weeks, resulted in enhanced functionality, including IFN-γ production after restimulation with cytokines or exposure to leukemia targets. This enhanced

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functionality extended to both primary NK cell subsets present in peripheral blood ($CD56^{bright}$ and $CD56^{dim}$). IL-15 was used as a survival cytokine during the in vitro rest period based on prior studies; however, additional cytokines that may contribute to the homeostasis and enhanced function of such cytokine-induced memory-like (CIML) NK cells have not been reported. Recent work has shown that murine IL-12, IL-15, and IL-18 preactivated NK cells have an enhanced ability to control tumor cell line challenge, which in vivo in mice required T cell–derived IL-2 [21].

We, therefore, investigated the expression of CD25, a key component required to form the high-affinity heterotrimeric IL-2Rαβγ on human NK cells briefly activated with combinations of IL-12, IL-15, and IL-18. NK cells constitutively express 2 components of the high-affinity IL-2R: the IL-2/15Rβ and γ_c, which form an intermediate affinity heterodimer that transduces signals in the presence of nanomolar concentrations of IL-2 or IL-15. In contrast, the heterotrimeric high-affinity IL-2Rαβγ (including IL-2Rα, CD25) is ligated by picomolar concentrations of IL-2, and thus CD25 expression dictates high-affinity IL-2 binding on NK cells [22,23]. Previous work has demonstrated constitutive, low-density expression of CD25 on $CD56^{bright}$ NK cells, facilitating cross talk with T cells and antigen presenting cells [24]. In addition, several studies have shown that IL-2 or IL-15 stimulation alone result in transient (hours to days) CD25 expression on the majority of NK cells [25,26]. Here, we report that short-term (16 hour) combined cytokine activation of human NK cells with IL-12, IL-15, and IL-18 induced robust and prolonged CD25 expression that persisted for at least 7 days on CIML NK cells. This CD25 expression resulted in a signal-competent high-affinity IL-2Rαβγ that stimulated STAT5 phosphorylation in NK cells. Moreover, picomolar concentrations of IL-2, acting via the IL-2Rαβγ costimulated IFN-γ production, enhanced cytotoxicity, and induced proliferation of NK cells. Furthermore, IL-12, IL-15, and IL-18 preactivated NK cells adoptively transferred into immunodeficient NOD-SCID-$\gamma_c^{-/-}$ (NSG) mice were selectively supported by exogenous rhIL-2. These findings provide a clear rationale for utilizing low-dose IL-2 therapy as 1 method to support CIML NK homeostasis and functionality cells after adoptive transfer of IL-12, IL-15, and IL-18 preactivated allogeneic NK cells.

MATERIALS AND METHODS

Reagents

The following antihuman mAbs were used: Beckman Coulter (Brea, California): CD56(N901), CD3(UCHT1); BD (Franklin Lakes, NJ): CD16(3G8), IFN-g(B27), CD25(M-A251), pSTAT5(pY694); purified anti-CD25 (B-B10, eBioscience, San Diego, CA) or isotype IgG1k (Biolegend, San Diego, CA). The following endotoxin-free cytokines were used: rhIL-2 (Chiron, Emeryville, CA); rhIL-15 (CellGenix, Freiburg, Germany); rhIL-12, rhIL-18 (Peprotech, Rocky Hill, NJ). The K562 (ATCC, Manassas, VA) cell line was maintained in RPMI-1640 plus 10% FCS and supplements FCS and supplements [20].

NK Cell Purification and Cell Culture

Anonymous human platelet apheresis donor peripheral blood mononuclear cells (PBMCs) were isolated by ficoll centrifugation. NK cells were purified to >95% $CD56^{+}CD3^{-}$ purity using Rosettesep enrichment (StemCell Technologies, Vancouver, Canada) as described [20]. In some experiments, $CD56^{dim}$ NK cells were isolated by flow cytometric sorting (>99% pure). NK cells were cultured at 5×10^6/mL in complete RPMI-1640 media (Hyclone, Logan, UT) with 10% human AB serum (Sigma, Saint Louis, MO) and supplements [20].

Assays to Assess CD25 Expression and Functions

Purified NK cells were preactivated for 16 hours with individual cytokines or combinations for 16 hours, washed 3 times, and replated in IL-15 (1 ng/mL) for 2 or 6 additional days. At each time point, cells were harvested and assessed for CD25 expression by flow cytometry. For pSTAT5 experiments, cells were stimulated with varying concentrations of IL-2 for 15 minutes and then stained for pSTAT5 as described [20]. In some experiments, cells were preincubated for 1 hour at 37°C with either an isotype or anti-CD25 antibody (10 μg/mL). Preactivated cells were restimulated after 3 days of in vitro culture with varying concentrations of IL-2, with or without IL-12. Cells were incubated for 6 hours in the presence of BFA/monensin (BD) and assayed for intracellular IFN-γ [20].

Flow Cytometric Analysis

Cell staining was performed as described [20,27], and data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo (TreeStar, Ashland, OR) or Kaluza (Beckman Coulter) software. Phospho-STAT5 flow cytometry assays were performed following the manufacturer's instructions (BD).

Flow-Based Killing Assay

Sorted $CD56^{dim}$ NK cells were preactivated for 16 hours, washed 3 times, and then cultured in cytokine-free media for 2 days. Cells were then cultured for an additional 24 hours, with or without the addition of IL-2, before harvesting for use as effectors in cytotoxicity assays. Flow-based killing assays were performed by coincubation with CFSE-labeled K562 cells for 4 hours and assaying 7-AAD uptake as described [28]. Spontaneous background K562 death (no effector control wells) was subtracted to yield percent specific killing, and in all cases was less than 5%. Flow-based killing assay results consistently correlate with ^{51}Cr release assays and provide a direct method to count individual dead target cells [28,29].

In Vitro Proliferation and Survival Assays

CFSE-labeled, sorted $CD56^{dim}$ NK cells were preactivated and cultured in IL-2–containing media, with media and cytokine changes every other day. On day 7, cells were harvested and assessed for CFSE-dilution staining or 7-AAD by flow cytometry.

Human NK Cell Xenograft Experiments

Purified human NK cells were preactivated with IL-12, IL-15, and IL-18 or 1 ng/mL IL-15 alone (control) for 16 hours and washed 3 times, and then equivalent numbers of control or CIML NK cells from the same donor were transferred retro-orbitally into groups of NSG mice (3×10^6/mouse to 8×10^6/mouse). Mice were injected with rhIL-2 (75,000 IU/mouse) every other day × 3 doses. On day 7, mice were sacrificed and human cells were identified with human CD45 and CD56/CD16 expression, and all human $CD45+$ cells were NK cells. The relative abundance of NK cells was determined by calculating the ratio of mouse CD45 to human CD45 positive cells in the spleen, to control for variable absolute leukocyte numbers in different NSG mice.

Statistical Analysis

Statistical comparisons were performed using Student *t*-test or ANOVA ($^{*}P < .05$; $^{**}P < .005$; $^{***}P < .0005$), where appropriate.

RESULTS

Induction of CD25 on IL-12, IL-15, and IL-18 Preactivated Human NK Cells

We hypothesized that combinations of IL-12, IL-15, and IL-18 stimulate NK cells to express CD25, thereby acquiring responsiveness to low concentrations of IL-2. To test this premise, cytokines known to bind to constitutively expressed cytokine receptors on resting NK cells were evaluated for the ability to induce cell surface CD25 expression. Purified human NK cells were cultured overnight in IL-2, IL-12, IL-15, IL-18, or combinations of these cytokines. Low-dose IL-15 (LD15) was used as a control to maintain survival. At 16 hours, all cytokines tested, with the exception of LD15, resulted in increased expression of CD25 on both $CD56^{dim}$ and $CD56^{bright}$ NK cells. However, these stimuli resulted in varying percentages of $CD25^{+}$ NK cells, which also varied markedly in expression density on a per cell basis (Figure 1). Stimulation with IL-2 or IL-12 alone modestly increased the percentage of $CD25^{+}$ NK cells, but the expression of per cell CD25 (MFI) was low (Figure 1B,C). IL-15 or IL-18 alone enhanced the percentage of NK cells expressing CD25, but again resulted in a small increase in per cell expression

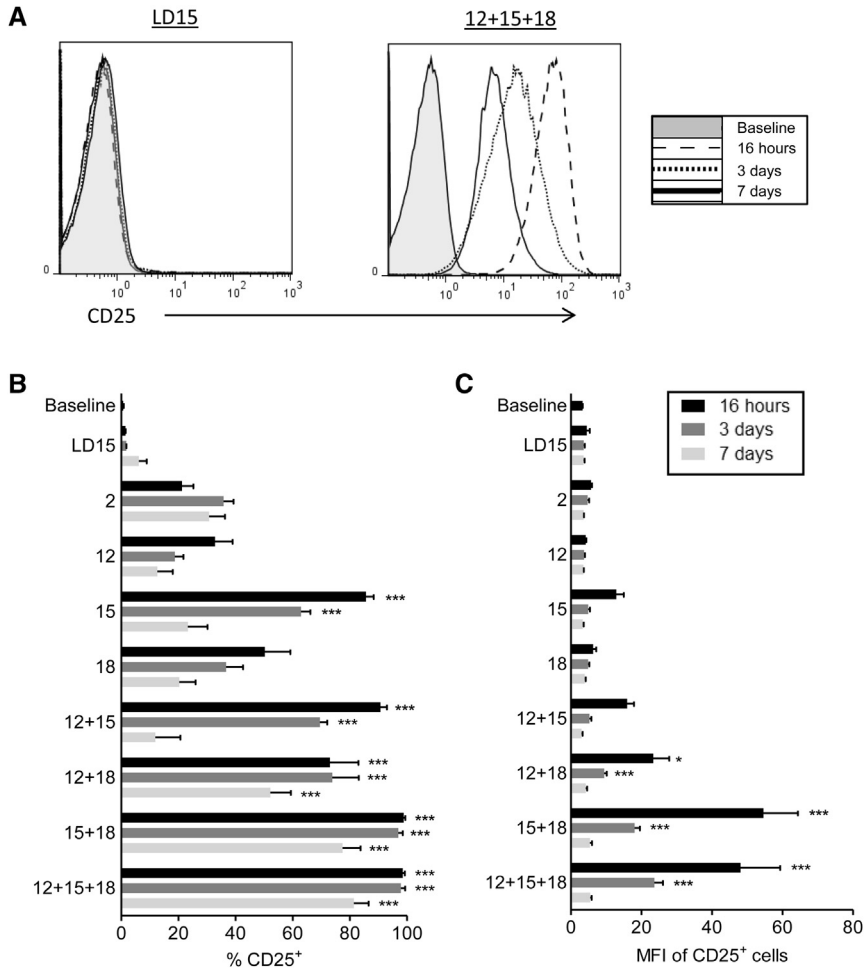

Figure 1. CD25 expression is induced on human NK cells by combined cytokine activation. Freshly purified human NK cells were stained for baseline CD25 expression and activated overnight with the following cytokines (1 ng/mL IL-15, LD15; 1 nM IL-2; 100 ng/mL IL-15; 10 ng/mL IL-12; 50 ng/mL IL-18) or combinations of the cytokines as indicated. After culture for 16 hours, cells were washed extensively and cultured for an additional 3 or 7 days in LD15 for all later time points. (A) Cell surface CD25 is induced after activation with IL-12, IL-15, and IL-18, compared with LD15, on NK cells. Representative flow histograms at baseline, 16 hours, 3 days, or 7 days after the initiation of IL-12, IL-15, and IL-18 preactivation or culture in LD15, demonstrating the marked increase and prolonged expression of CD25. (B and C) CD25 percentage (B) and mean fluorescence intensity (MFI) (C) of NK cells at various time points after cytokine activation. Summary data represent n = 6 donors (3 independent experiments). Data shown are mean ± SEM of percentage or the MFI of $CD25^+$ NK cells. Significance is shown comparing each time point versus fresh (baseline) NK cells, and was calculated by 1-way ANOVA. These data show that although individually IL-2, IL-12, IL-15, and IL-18 induce $CD25^+$ $CD56^{dim}$ NK cells, the combinations of IL-15+IL-18 and IL-12+IL-18 result in higher per cell CD25 expression.

(mean fluorescence intensity [MFI]). The combination of IL-15 and IL-18, with or without IL-12, resulted in the highest induction of CD25 per cell on NK cells (16-fold increase in MFI, Figure 1A-C). Withdrawal of cytokine stimulation after 16 hours resulted in decreased CD25 expression over the course of 7 days, although a large fraction of $CD25^+$ cells persisted after preactivation with IL-15 plus IL-18 or IL-12 plus IL-18, or IL-12 plus IL-15 plus IL-18, stimuli that have previously been reported to induce human CIML NK cells [20]. We also observed increased expression of CD25 by both $CD56^{bright}$ and $CD56^{dim}$ NK cells, but notably, the MFI increase in $CD56^{dim}$ NK equaled or exceeded that of $CD56^{bright}$ NK cells in every cytokine condition tested (data not shown). Similar results were seen with NK cells cultured within PBMC (Supplemental Figure 1). Because CD25 is not expressed in freshly isolated $CD56^{dim}$ NK cells, and is at a low density on $CD56^{bright}$ NK cells, we evaluated whether the mechanism of induction of CD25 was at the IL2RA (CD25) mRNA level. There was a marked increase in IL2RA mRNA in NK cells after stimulation (Supplemental Figure 2), suggesting that the mechanism of increased CD25 expression is primarily to enhance transcription or stability of CD25 mRNA. Thus, NK cells preactivated with the combination of cytokines that induce CIML NK cells also express CD25 for a prolonged time period, which may facilitate responsiveness to low concentrations of IL-2.

CD25 Induced on NK Cells by Cytokines Results in a Signal-Competent IL-2Rαβγ

CD25 associates with the intermediate-affinity IL-2Rβγ subunits to form the high-affinity heterotrimeric IL-2Rαβγ [22,30]. In response to ligation with IL-2, this complex signals through the IL-2Rβγ chains, resulting in phosphorylation of STAT5 [22,23]. We, therefore, evaluated whether cytokine-activated NK cells exhibited increased phospho-STAT5 when stimulated with picomolar concentrations of IL-2, which selectively ligate the high-affinity IL-2Rαβγ. In these experiments, we focused on preactivation cytokine combinations that result in CIML NK cells and the most pronounced induction of CD25: IL-15 plus IL-18 (also allowing assessment

of IL-12-independent responses), or IL-12 plus IL-18 (Figure 1). NK cells were preactivated for 16 hours with low doses of IL-15 (LD15, control), IL-12 plus IL-18, or IL-15 plus IL-18, washed, and rested in medium alone for an additional 2 days to allow the NK cells to return to a baseline state, but with maintained expression of CD25 (Figure 1). These rested NK cells were then evaluated for their ability to phosphorylate STAT5 in response to varying concentrations of IL-2. In these assays concentrations of 10 to 100 pM, IL-2 selectively activate IL-2Rαβγ, whereas 1 nM IL-2 also activates the constitutively expressed intermediate affinity IL-2Rβγ [23]. NK cells that were preactivated with IL-12 plus IL-18 or IL-15 plus IL-18 demonstrated increased STAT5 phosphorylation beginning at 10 pM IL-2, compared with ≥ 100 pM IL-2 required to induce pSTAT5 in control LD15 preactivated NK cells (Figure 2). Thus, cytokine-induced CD25 expression on NK cells results in signaling in response to picomolar concentrations of IL-2, indicating the presence of signal-competent heterotrimeric IL-2Rαβγ complexes. We next evaluated whether such signals result in enhanced NK cell function.

Signals via the Induced IL-2Rαβγ on Costimulate IFN-γ Production

In resting CD56^{bright} NK cells, CD25 is required for picomolar concentrations of IL-2 to act in concert with IL-12 for IFN-γ production [24]. In contrast, resting CD56^{dim} NK cells fail to produce IFN-γ in response to picomolar concentrations of IL-2 plus IL-12. Because both CD56^{bright} and CD56^{dim} NK cells exhibit memory-like properties, we evaluated the ability of low-dose IL-2 to costimulate IFN-γ production in these NK cell subsets after combined cytokine induction of CD25. In these experiments, purified NK cells were preactivated with cytokine combinations that result in CIML NK cells [20] and CD25 induction (IL-12 plus IL-18 or IL-15 plus IL-18) for 16 hours, washed, and then rested for 2 days in cytokine-free media to allow the NK cells to return to a baseline state (not producing IFN-γ protein). In the setting of

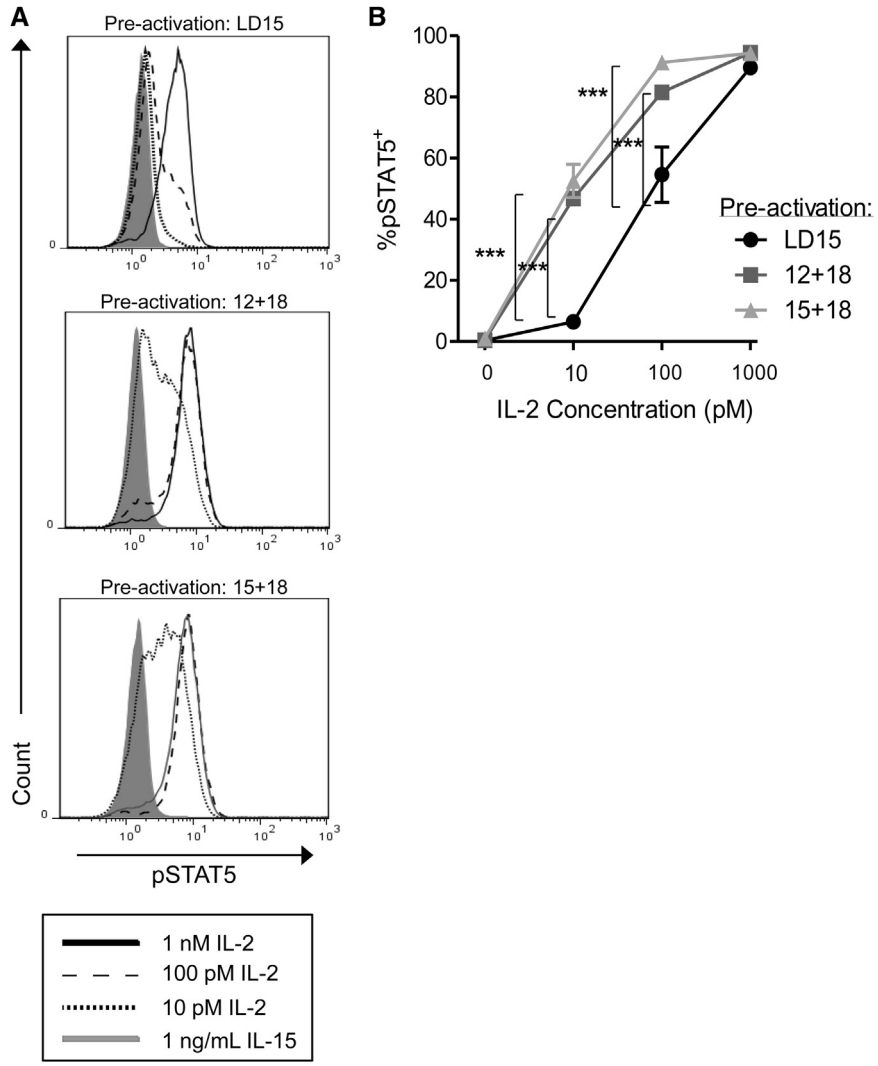

Figure 2. Picomolar concentrations of IL-2 induce phospho-STAT5 expression in preactivated NK cells. Purified NK cells were preactivated with LD15 (control), IL-12+IL-18, or IL-15+IL-18, washed extensively, and replated in cytokine-free media for 2 days to allow recovery to a resting state. IL-2 was added at the indicated concentrations for 15 minutes and levels of phospho-STAT5 were analyzed by intracellular flow cytometry. Shown is 1 representative donor (A), or summary data of the mean ± SEM percentage pSTAT5 positive CD56^{dim} NK cells from n = 3 donors and experiments (B). Preactivated NK cells respond to 10 and 100 pM IL-2 with enhanced STAT5 phosphorylation over control. Significance was calculated by ANOVA.

cytokine preactivation, we observed a moderate fraction of NK cells producing IFN-γ in response to IL-2 alone, indicating that prior cytokine activation primes an enhanced IFN-γ response to individual cytokines (Supplemental Figure 3) [20]. Similarly, a modest fraction of preactivated NK cells produce IFN-γ in response to IL-12 alone, again due to the initial preactivation (0 pM IL-2, Figure 3C). When IL-2 and IL-12 were combined, concentrations of 10 or 100 pM IL-2 were able to significantly costimulate IFN-γ production in cytokine-preactivated NK cells. Concentrations above 100 pM failed to further increase IFN-γ production, suggesting that additional signaling via intermediate affinity IL-2Rβγ

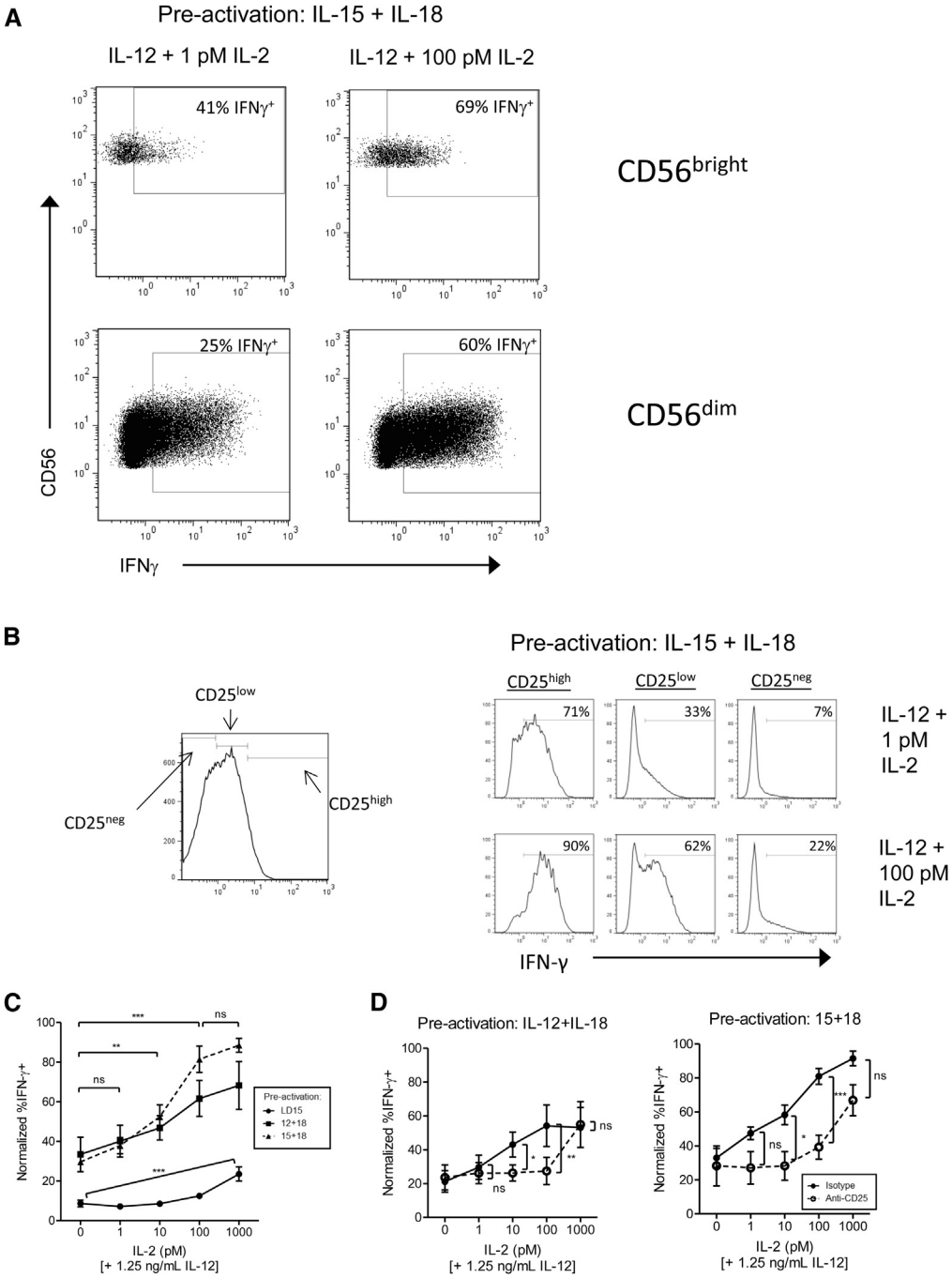

Figure 3. Induced CD25 on preactivated NK cells results in a functional high-affinity IL-2Rαβγ that signals for enhanced IFN-γ production. Purified NK cells were activated for 16 hours with IL-12+IL-18 or IL-15+IL-18, washed extensively, and replated in cytokine-free media for 2 days to allow recovery to a resting state. IL-2 was then added at the indicated doses in combination with IL-12 (1.25 ng/mL). After 6 hours, IFN-γ was measured by intracellular flow cytometry. (A) Representative flow cytometric plots from 1 preactivated donor depicting dose-dependent IFN-γ production costimulated by picomolar IL-2. (B) Representative flow plots from IL-15+IL-18 preactivated NK cells that were subgated based on CD25 expression and IFN-γ expression with stimulation by IL-12 + 1 or 100 pM IL-2. (C) Summary data of preactivated $CD56^{dim}$ NK cell IFN-γ production with IL-12 combined with various doses of IL-2, shown as mean ± SEM normalized IFN-γ percentage (n = 4 donors). (D) In a separate assay, cells were pretreated for 1 hour with either an anti-CD25–blocking antibody or IgG1 isotype control, followed by stimulation with 1.25 ng/mL IL-12 and 10-fold dilutions of IL-2. In (B,C), data are shown with the maximum IFN-γ positive percentage in each donor (n = 4) normalized to 100%, as absolute $IFN\text{-}\gamma^+$ percentages between donors are variable (range of maximum $\%IFN\text{-}\gamma^+$:14% to 66% for IL-12+IL-18 preactivated cells, 28% to 86% for IL-15+IL-18 preactivated cells). Significance was calculated by ANOVA.

was not required for maximal IFN-γ in this setting. Control NK cells that were not preactivated with cytokines but were cultured in medium alone (ie, 72 hours in medium only) excluded trypan blue and 7-AAD but failed to produce IFN-γ, even in response to established costimulatory conditions in resting CD56^{dim} NK cells (eg, 1 nM IL-2 plus IL-12) [31], likely due to prolonged growth factor starvation (data not shown). Therefore, we cultured NK cells in low-dose IL-15 (1 ng/mL), which preserves the functionality of these cells but does not induce CD25 expression (Figure 1B,C). Low-dose IL-15–activated cells did not produce increased IFN-γ with addition of IL-12 and IL-2, with the exception of 1nM IL-2 (Figure 3C). Thus, low concentrations of IL-2 stimulate and costimulate (in cooperation with IL-12) IFN-γ production by combined cytokine-preactivated NK cells. We next evaluated the absolute requirement for CD25 and the high-affinity IL-2Rαβγ for these IL-2 effects.

IFN-γ Production in Response to Picomolar Concentrations of IL-2 Requires CD25 and the High-affinity IL-2Rαβγ

Selective responsiveness to 10 to 100 pM IL-2 suggests action through the high-affinity IL-2Rαβγ. Furthermore, increased CD25 induction mediated by IL-15+IL-18 versus IL-12+IL-18 enhanced IL-2–costimulated IFN-γ production (mean 53% versus 32%, $P < .05$), and populations that expressed more CD25 per cell also produced more IFN-γ (Figure 3B). However, to definitively establish the requirement for CD25 and high-affinity binding, we used a blocking anti-CD25 mAb. This approach selectively blocks the formation of the high-affinity IL-2Rαβγ because CD25 is not available to the complex, but it allows stimulation via the intermediate-affinity IL-2Rβγ. The enhanced IFN-γ production in response to 10 or 100 pM IL-2 by cytokine-preactivated NK cells was abrogated by preincubation with an anti-CD25 mAb (Figure 3D). In contrast, 1 nM IL-2 costimulated IFN-γ production regardless of CD25 blockade. Collectively, these data indicate that picomolar concentrations of IL-2, signaling through an induced IL-2Rαβγ, stimulate enhanced IFN-γ in preactivated NK cells.

Picomolar Concentrations of IL-2 Act via IL-2Rαβγ to Augment Cytotoxicity by Cytokine-Preactivated NK Cells

Resting CD56^{dim} NK cells mediate cytotoxicity when triggered by the appropriate target cell [1,32]. We hypothesized that similar to the IFN-γ results, picomolar concentrations of IL-2 would augment preactivated NK cell cytotoxicity. To investigate this, we preactivated CD56^{dim} NK cells with low-dose IL-15, IL-12 plus IL-18, or IL-15 plus IL-18 for 16 hours; rested these cells in medium only (without IL-2) for 48 hours; and then stimulated them with varying concentrations of IL-2. After 24 hours, a standard flow-based cytotoxicity assay was performed with K562 leukemia cells as targets. Consistent with observations from the IFN-γ experiments, we observed significantly enhanced killing with 10 pM IL-2 stimulation, an effect that was prevented by preincubation with an anti-CD25 antibody (Figure 4). Notably, incubation with 1 nM IL-2 failed to enhance cytotoxicity above levels induced by 10 pM IL-2 in preactivated CD56^{dim} NK cells, consistent with expression of IL-2Rαβγ and saturation of IL-2 signaling. Low-dose IL-15–activated NK cells had enhanced killing only when cultured with 1 nM IL-2. Importantly, CD25 blockade abrogated enhanced cytotoxicity in response to picomolar concentrations of IL-2, but not at 1 nM, suggesting that the intermediate-affinity IL-2Rβγ was being utilized at this IL-2 concentration. In each preactivation condition, the observed killing was dependent on the effector to target ratio, as expected (Figure 4D,E and Supplemental Figure 4).

Low Concentrations of IL-2 Facilitate Cytokine-activated NK Cell Proliferation via the IL-2Rαβγ

T cell secretion of IL-2 acts in an autocrine or paracrine capacity to induce activated T cell proliferation [23]. Additionally, activated mouse NK cells, either in the context of in vivo viral infection or in vitro cytokine stimulation, undergo substantial proliferation [4,33]. Resting CD56^{dim} NK cells have been characterized as less proliferative compared with CD56^{bright} NK cells after cytokine stimulation [1,34-36]; however, proliferation by CD56^{dim} NK cells after combined cytokine preactivation has been observed [20]. We evaluated whether combined cytokine-activated CD56^{dim} NK cells proliferated in response to low doses of IL-2 using the high-affinity IL-2Rαβγ. Flow-sorted CD56^{dim} NK cells were labeled with CFSE and preactivated with low-dose IL-15, IL-12 plus IL-18, or IL-15 plus IL-18. After 16 hours, the cells were washed and incubated with anti-CD25 or isotype control mAbs for 1 hour. After this blockade, IL-2 was supplemented at the indicated concentrations. In medium only (without IL-2, IL-15, or other cytokines), we observed minimal proliferation (Figure 5A). However, with IL-2 added at 10 pM or greater, CD56^{dim} NK cells were induced to proliferate in a dose-dependent manner (Figure 5A-C). Furthermore, when cells were precultured with an anti-CD25–blocking antibody, the proliferation induced by low-dose IL-2 (10 to 100 pM) was abrogated (Figure 5B,C). We observed substantial donor-dependent variability in the degree of proliferation by CD56^{dim} NK cells as demonstrated in Figure 5B and C, presumably due to reported differences in the proliferative capacity of human NK subsets [37-39]. However, all donors displayed a significant enhancement in proliferation with $\geq$10 pM IL-2 stimulation, as well as abrogation of this signaling after CD25 blockade.

IL-2 Supports Cytokine-Preactivated CIML NK Cells in vivo in NSG Mice

Preactivation of human NK cells with IL-12, IL-15, and IL-18 for 16 hours followed by prolonged culture in vitro with IL-15 for survival results in CIML NK cells. Based on the induction of a functional IL-2Rαβγ on preactivated NK cells, we hypothesized that rhIL-2 would selectively support cytokine-preactivated (IL-12, IL-15, IL-18) compared with control- (IL-15 only) activated NK cells in vivo. Cytokine-preactivated or control NK cells were generated from the same donor and, after extensive washing, were transferred into NSG mice, and rhIL-2 was administered every other day. After 1 week, mice were analyzed for human NK cell numbers and evaluated for CIML NK cell recall responses. Cytokine-preactivated NK cells had significantly improved engraftment in NSG mice in this system, compared with control (IL-15) NK cells (Figure 6). Both CD56^{bright} and CD56^{dim} NK cells were identified, and enhanced recall functionality to cytokine or tumor target–based restimulation by CIML NK cells was also evident ex vivo. These data suggest that administration of rhIL-2 after adoptive transfer of IL-12, IL-15, and IL-18–preactivated NK cells is a novel approach to administer and support function-enabled NK cells as adoptive immunotherapy for patients with hematologic malignancies.

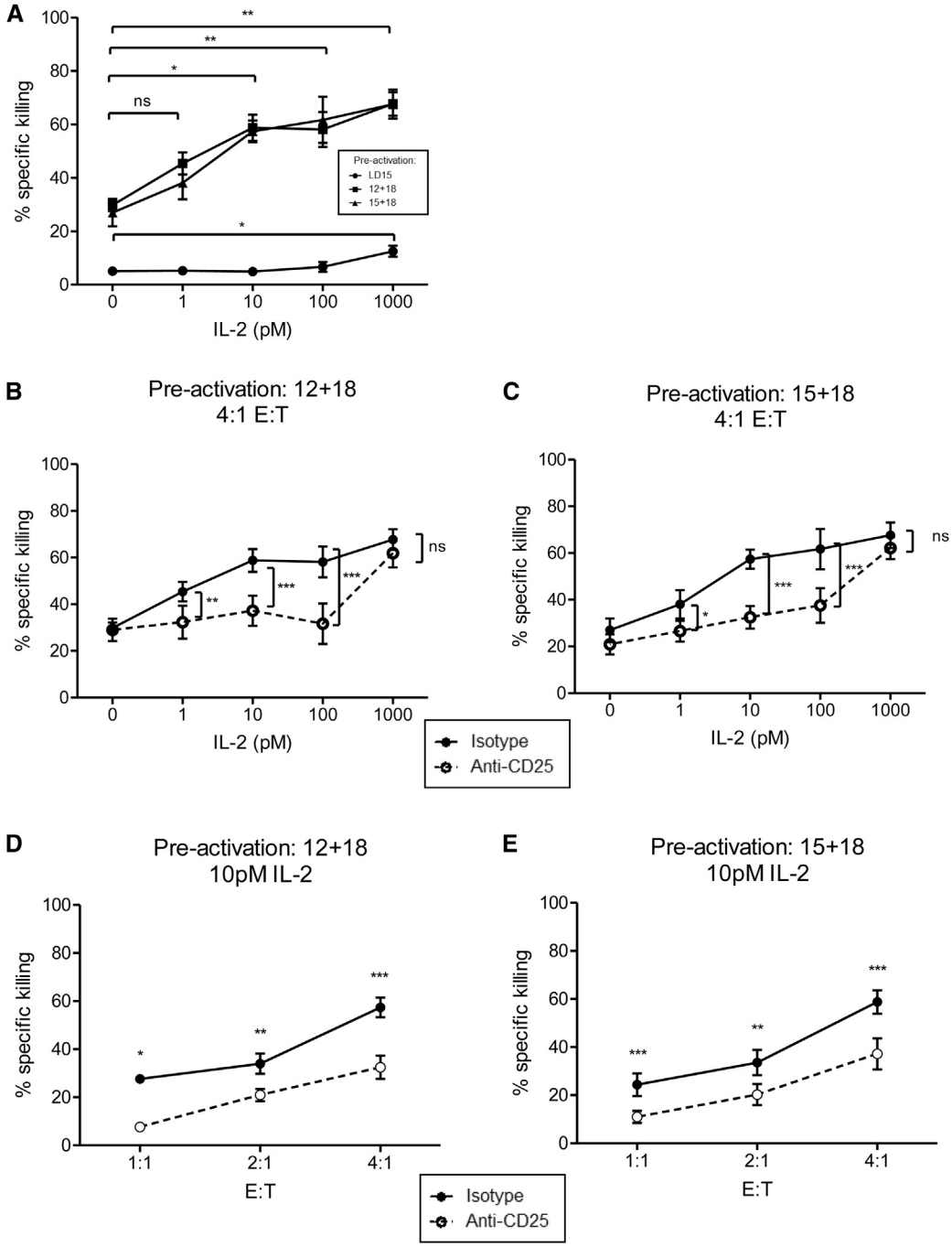

Figure 4. Low concentrations of IL-2 enhance preactivated CD25^{+} CD56^{dim} NK cell killing of K562 leukemia target cells. Flow-sorted CD56^{dim} NK cells were preactivated with LD15, IL-12+IL-18, or IL-15+IL-18, washed, and replated in cytokine-free media for 2 days. Cells were then incubated for 1 hour with an isotype or anti-CD25–blocking antibody before activation with dilutions of IL-2. After 24 hours, cells were harvested and plated in a 4-hour in vitro flow-based killing assay with CFSE-labeled K562 cells. Shown is mean ± SEM percent specific killing at a 4:1 effector to target ratio preincubated with (A) an isotype control or (B,C) an anti-CD25–blocking antibody. (D,E) Killing by preactivated CD56^{dim} NK cells is NK cell dose- (effector to target ratio) dependent, shown at 10 pM IL-2, with anti-CD25 or isotype control preincubation. Data are summarized from 3 to 5 donors in 2 independent experiments.

DISCUSSION

Freshly isolated human CD56^{dim} NK cells are thought to require high concentrations of IL-2 (or IL-15), signaling through the IL-2/15Rβγ, to enhance or prime their functional capacity through this receptor complex. Here, we show that combined cytokine activation of both CD56^{bright} and CD56^{dim} NK cells with IL-15 plus IL-18 or IL-12 plus IL-18 results in robust CD25 expression, which remains present on CIML NK cells after 7 days. This CD25 expression combines with constitutively present IL-2/15Rβ and γ_c to form a functional high-affinity IL-2Rαβγ on preactivated NK cells. Such NK cells have increased STAT5 phosphorylation when restimulated with picomolar concentrations of IL-2 and exhibit augmented functional responses, including IFN-γ production, cytotoxicity, and proliferation. Further, IL-2 supports cytokine-preactivated NK cells in vivo in NSG xenograft

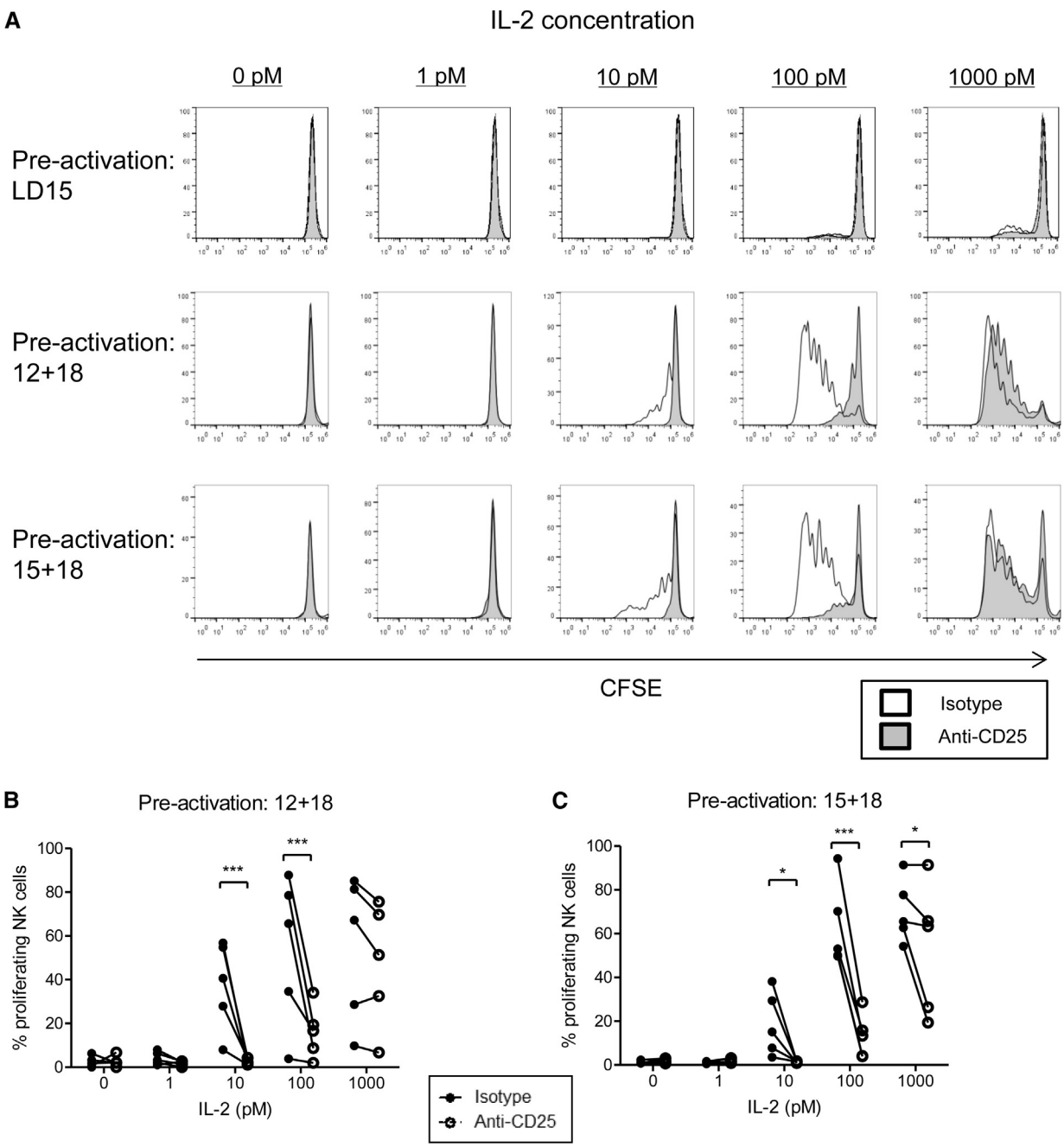

Figure 5. Low concentrations of IL-2 stimulate the proliferation of preactivated CD56^{dim} NK cells. CFSE-labeled, flow-sorted CD56^{dim} NK cells were preactivated with low-dose IL-15, IL-12+IL-18, or IL-15+IL-18 for 16 hours, washed extensively, and blocked with an isotype or anti-CD25–blocking antibody. Dilutions of IL-2 were then added, with subsequent media, cytokine, and blocking antibody additions occurring every other day. After 7 days, cells were harvested and analyzed for CFSE dilution by flow cytometry. Analysis was restricted to live cells by 7-AAD exclusion. (A) Data shown represent histograms from 1 donor. Both isotype-blocked (open histograms) and anti-CD25–blocked (shaded histograms) NK cell proliferation are shown. (B and C) Summary data depicting the variability of proliferation between IL-12+IL-18 or IL-15+IL-18 activated CD56^{dim} NK cells from different donors and the dependence of picomolar IL-2 mediated proliferation on CD25/IL-2Rαβγ. Significance was assessed by 2-way ANOVA.

models. Thus, both primary peripheral blood NK cell subsets (CD56^{bright} and CD56^{dim}) are induced to express IL-2Rαβγ after combined cytokine preactivation, expanding the potential of low concentrations of IL-2 to stimulate and support CIML NK cells. These data suggest that low-dose IL-2 therapy can support survival, proliferative, and effector functions of IL-12, IL-15, and IL-18–preactivated allogeneic NK cells after adoptive transfer.

What role does the high-affinity IL-2Rαβγ on CD56^{dim} NK cells play during a physiologic immune response? During the host response to a pathogen, NK cells have a complex set of interactions with other members of the innate and adaptive immune system [2,40,41]. After infection with MCMV, a model virus infection with a prominent early NK cell response, an initial wave of cytokines are produced that drive NK cell proliferation and enhances their functional capacity. In this setting, IL-12, IL-15, and IL-18 are induced over 1 to 2 days, a time frame consistent with the induction of CD25 on mouse NK cells [42,43]. In a recent report from the Biron laboratory, induced CD25 was identified on NK1.1^{+}TCRβ^{-} NK

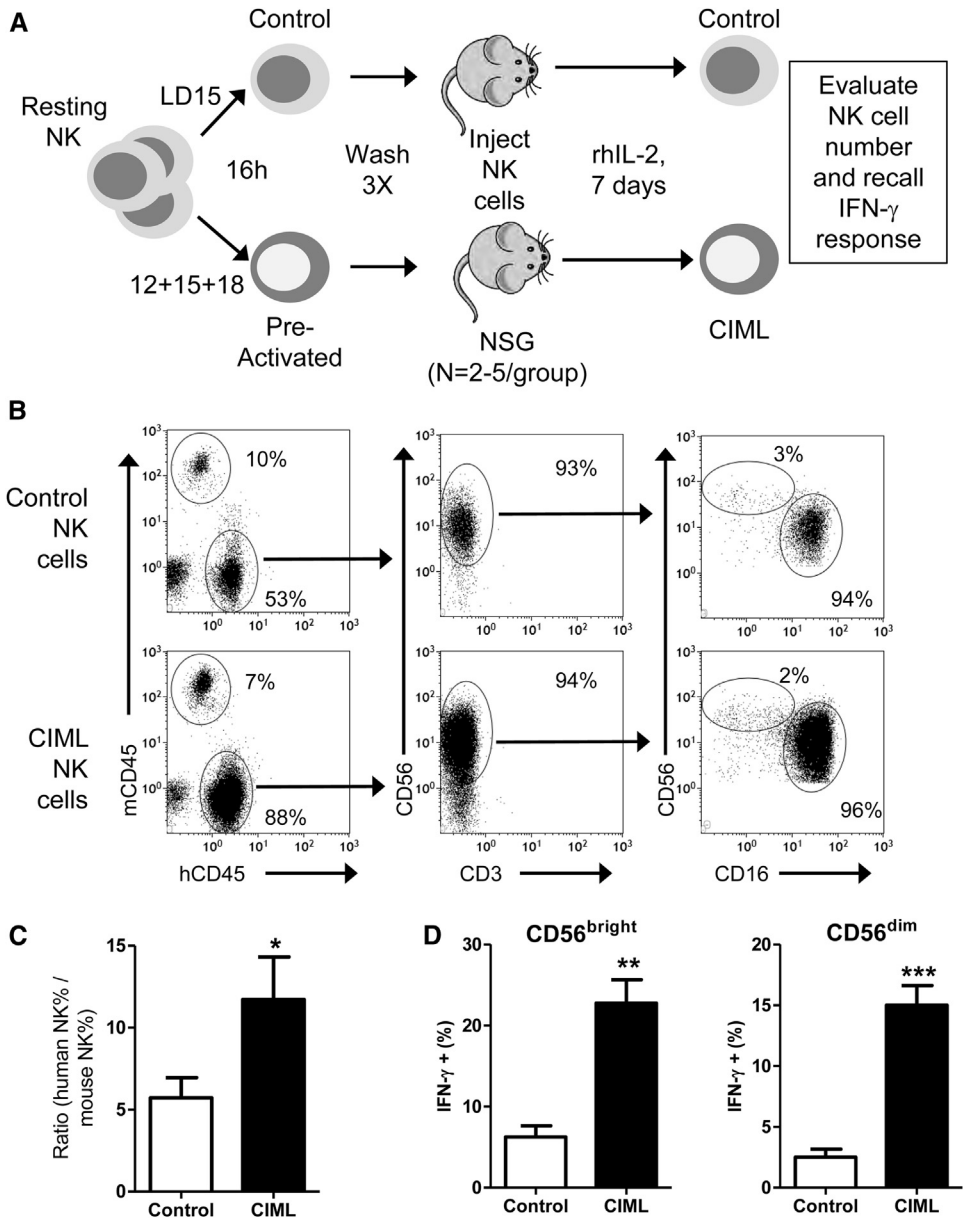

Figure 6. Exogenous IL-2 supports CIML NK cells in a NSG xenograft model. (A) Purified human NK cells were preactivated with IL-12, IL-15, and IL-18 (CIML) or low-dose IL-15 only (control) for 16 hours, washed, and identical numbers of NK cells were transferred into NSG mice, followed by injections of 75,000 IU i.p. of rhIL-2 every other day. After 7 days, mice were assessed for human NK cell content and CIML NK cell recall responses. (B) Representative flow plots from the peripheral blood of mice injected with the same number of CIML or control NK cells from the same donor 7 days earlier. Human $CD45^+$ cells are $CD56^+CD3^-$ NK cells with preserved $CD56^{bright}$ and $CD56^{dim}$ NK cells subsets. (C) Summary data from 3 different NK cell donors transferred into a total of 12 NSG mice demonstrating superior engraftment of CIML, compared with control NK cells. The ratio of human to mouse CD45+ cells, representing the relative abundance of human NK cells, is used to control for differences in blood volume obtained. (D) Mouse splenocytes containing adoptively transferred human NK cells were evaluated for a recall IFN-γ response after IL-12+IL-15 restimulation, demonstrating that CIML NK cells exhibit preserved, enhanced functionality in this xenograft model. Significance was assessed by *t*-test, with $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

cells peaking approximately 3.5 days after MCMV infection. In vitro, NK cells within bulk murine splenocytes were induced to express CD25 after 24 hours by the combination of IL-12 plus IL-18, which absolutely required IL-12-induced STAT4 signals [43]. Thus, we suspect that during human responses to viruses and potentially other pathogens, IL-12, IL-15, and IL-18 are produced, and $CD56^{dim}$ NK cells are induced to express CD25 (Supplemental Figure 5). In our studies, we identified that human NK cells are optimally stimulated by IL-15 plus IL-18 (in the presence or absence of IL-12) to express CD25, differing from mouse NK cell requirement for IL-12. Because all doublet combinations of IL-12, IL-15, and IL-18 resulted in CD25 expression, human NK cells may have an expanded flexibility to express the high-affinity IL-2Rαβγ in different inflammatory cytokine responses. Notably, IL-18 appeared to be necessary, but not sufficient, for substantial CD25 expression in our experiments.

Recently, combined cytokine preactivation with IL-12, IL-15, and IL-18 has been shown in mouse [16,21,44] and human [20] NK cells to result in memory-like NK cell functions, with long-lived, enhanced functionality to restimulation. CIML NK cell function refers to NK cells that are preactivated (eg, IL-

12+IL-15+IL-18), return to a resting baseline state, and then exhibit enhanced functional responses to a wide variety of restimulation triggers, including cytokines, activating NK cell receptors, or tumor cells. This was first described in mouse systems [16,45], and human NK cells were also found to exhibit similar CIML responses [20]. In addition, Ly49H-mediated "memory" NK cell function in the context of MCMV infection was found to depend on early proinflammatory cytokine signals [17]. For human NK cells, it is noteworthy that the same cytokine combinations of IL-12 plus IL-18 or IL-15 plus IL-18 that preactivate memory-like NK functionality are also the optimal cytokine inducers of CD25 expression. Although human CIML NK cells were supported with IL-15 in vitro after preactivation [20], low concentrations of IL-2 may also have a role in supporting these memory-like NK cell functions, either endogenously or exogenously [21]. In addition, mouse IL-12, IL-15, and IL-18–preactivated NK cells were also recently shown to effectively clear mouse tumor cell line challenges in vivo [21]. After transfer into irradiated syngeneic mice, NK cells were found to require $CD4^+$ T cell derived IL-2 in vivo to effectively eliminate lymphoma and melanoma cell line challenges. Both murine and human NK cells were also shown to express CD25 immediately after IL-12, IL-15, and IL-18 activation, which is consistent with our findings in human NK cell subsets [21]. Thus, both human and mouse NK cells can be induced to express CD25 and a high-affinity IL-2Rαβγ, which may be important to support NK cell functional competency after viral infection or after exogenous IL-12, IL-15, and IL-18 preactivation.

What are the clinical implications of peripheral blood NK cell acquisition of a functional high-affinity IL-2Rαβγ? Allogeneic NK cell therapy has evidence of antileukemic activity in clinical trials in acute myelogenous leukemia patients [46]. Low-dose or ultra-low–dose IL-2 therapy has been shown to expand $CD56^{bright}$ NK cells selectively over $CD56^{dim}$ NK cells in patients with HIV infection or cancer [47,48], most likely due to lack of the high-affinity IL-2Rαβγ on $CD56^{dim}$ NK cells that promotes survival in the resting state [49]. As $CD56^{dim}$ NK cells represent 80% to 95% of the mature peripheral blood NK population, adoptive transfer of NK cells preactivated with combinations of IL-12, IL-15, and IL-18, followed by low-dose IL-2 therapy in vivo, may be a useful strategy to further enhance antitumor and/or antiviral NK cell responses. Such an approach would include strategies to limit regulatory T cell numbers or function.

In summary, NK cells that are preactivated with combinations of IL-12, IL-15, and IL-18 are induced to express CD25 and a high-affinity IL-2Rαβγ, which persists in CIML NK cells. This results in an acquired ability of all peripheral blood NK cells to proliferate, kill tumor targets, and produce IFN-γ in response to picomolar concentrations of IL-2. IL-2 supports CIML NK cells in vivo in xenograft models. Thus, our findings provide a rationale for low-dose IL-2 therapy to enhance expansion and NK cell function after IL-12, IL-15, and IL-18–preactivated NK cell adoptive transfer as immunotherapy for cancer patients.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.01.006.

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